Int J Clin Exp Pathol 2018;11(4):2193-2200
www.ijcep.com /ISSN:1936-2625/IJCEP0074657

Original Article

Low nuclear zinc finger and BTB domain containing 7A expression is an independent prognostic factor for recurrence-free survival in invasive ductal carcinoma of the breast

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Received February 17, 2018; Accepted March 24, 2018; Epub April 1, 2018; Published April 15, 2018

Abstract: The role of zinc finger and BTB domain containing 7A (ZBTB7A) in oncogenesis has been shown to be context-dependent, participating in pro-oncogenic or oncosuppressive mechanisms by directly regulating gene transcription or by interacting with other regulatory proteins. Alterations in ZBTB7A expression have been associated with worse prognosis. We examined ZBTB7A protein expression in breast carcinoma tissue samples and analyzed its clinical and prognostic significance. Tissue microarray blocks from 196 cases of invasive ductal carcinoma (IDC) were immunostained and <65% positively stained tumor cell nuclei were defined as low ZBTB7A expression. Of 196 IDC cases, 120 (61.2%) showed low ZBTB7A expression. Low nuclear ZBTB7A expression was associated with larger tumor size, higher histological grade, estrogen receptor negativity, progesterone receptor negativity, triple negativity, and recurrence. Cytoplasmic ZBTB7A expression was not associated with any clinicopathological characteristics. In univariate survival analysis, nuclear ZBTB7A expression did not affect overall or recurrence-free survival. However, multivariate survival analysis revealed that ZBTB7A independently predicted recurrence-free survival of IDC patients. Reduced ZBTB7A expression is associated with aggressive oncogenic behavior of IDC. ZBTB7A expression may be a novel prognostic biomarker for predicting recurrence-free survival of IDC patients.

Keywords: Breast, invasive ductal carcinoma, zinc finger and BTB domain containing 7A, immunohistochemistry, prognosis

Introduction

Zinc finger and BTB domain containing 7A (ZBTB7A; also known as factor that binds to inducer of short transcripts protein 1, Pokemon, leukemia/lymphoma-related factor, and osteoclast-derived zinc finger) is a member of the BTB/poxvirus and zinc finger, and Kruppel (POK) family of transcription factors. Through the zinc finger domain, POK proteins bind to a specific consensus sequence in the target gene promoter to regulate transcription. The BTB domain enables homodimer formation and interaction with co-repressor proteins such as BCL6, a silencing mediator of retinoid and thyroid receptors, and mSin3A which is a histone deacetylase that contributes to transcriptional repression by POK proteins [1, 2]. For ZBTB7A, the list of BTB domain-interacting proteins includes BCL6, specificity protein 1, and androgen receptor [2]. Being a transcription factor and having a nuclear localization signal at the C-terminal, ZBTB7A is often detected predominantly in the nucleus [3-8].

ZBTB7A plays developmental roles as demonstrated in the differentiation of hemato-lymphoid cells, chondrocytes, adipocytes, and osteoclasts [1, 2]. Moreover, *ZBTB7*A mRNA and protein A have been identified in both normal and cancerous tissues of multiple organs. Increased ZBTB7A expression has been recognized in diffuse large B-cell lymphoma and carcinomas of the colon, stomach, lung, ovary,

liver, prostate, and breast [4, 5, 7, 9-12]. A negative prognostic effect of high ZBTB7A expression has been reported in carcinomas of the stomach, lung, ovary, liver, and breast carcinomas, suggesting a tumor-promoting function of ZBTB7A [7, 10, 11, 13-15]. On the other hand, reduced ZBTB7A expression has been associated with worse prognosis in colorectal carcinoma, malignant melanoma, diffuse large B-cell lymphoma, and acute myeloid leukemia, implicating its tumor-suppressive role [12, 16-18]. The mechanism of ZBTB7A's action appears to be versatile depending on the cell type. For example, ZBTB7A is oncogenic by transcriptionally repressing the tumor suppressor alternative reading frame in lymphoma cells and by transcriptionally activating membrane type 1 matrix metalloproteinase in ovarian carcinoma cells [10, 12]. The tumor-suppressive function of ZBTB7A occurs through transcriptional repression of glycolytic genes and melanoma cell adhesion molecule (MCAM) or through indirect inhibition of a SOX9-dependent oncogenic pathway [16-19]. Deletion and inactivating mutations [8, 16, 18], as well as suppression by microRNA [11, 19, 20], have been suggested to contribute to the reduced ZBTB7A expression.

Three previously published reports describe ZBTB7A expression in breast carcinoma [4, 14, 15]. In two of these reports, ZBTB7A protein was overexpressed in a subset of invasive ductal carcinomas (IDCs) and associated with a worse prognosis [14, 15]. We revisited the issue with our cohort of 196 IDC cases, giving a detailed examination on the immunohistochemical expression of ZBTB7A and analyzing its possible associations with clinicopathological characteristics and patient outcome.

Materials and methods

Patient specimens and tissue microarray construction

The breast cancer tissue microarray used in this study has been previously described [21]. Briefly, the surgically resected specimens were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin using a standard protocol. All available hematoxylin and eosin-stained slides were reviewed by two Board-certified pathologists specialized in breast pathology, and the two most representative tumor areas were carefully selected and marked on individual formalin-fixed, paraffin-embedded tissue blocks. Two tumor tissue cores (2 mm in diameter) were then obtained from each specimen and manually arrayed in recipient paraffin blocks. The tissue microarray consisted of 196 IDC cases. Nine cases were recurrent tumors, which were excluded from the survival analysis. The recurrence and survival status was updated by electronic medical record review for each included patient as of September 1, 2016. The histological grade of IDC had been determined according to the modified Bloom-Richardson grading system. The stage was based on the 7th American Joint Committee on Cancer staging manual. The study (2016-06-036) was reviewed and approved by the Institutional Review Board of the Kangbuk Samsung Hospital (Seoul, Republic of Korea).

Immunohistochemistry

Immunohistochemical staining was performed on 3 μm-thick tissue microarray block sections. Briefly, the sections were dehydrated and deparaffinized in xylene and then rehydrated in a graded series of alcohol solutions. We used primary antibodies against ZBTB7A (1:200, polyclonal; Novus Biologicals, Littleton, CO, USA), estrogen receptor (ER; 1:200, clone SP1; LabVision Corporation, Fremont, CA, USA), progesterone receptor (PR; 1:200, clone PgR 636; DakoCytomation, Glostrup, Denmark), and human epidermal growth factor receptor 2 (HER2; 1:200, clone SP3, LabVision Corporation). Immunostaining was performed using a compact polymer method (Bond Intense Detection Kit; Leica Biosystems, Newcastle upon Tyne, UK). The primary antibodies were detected with Dako EnVision+ Systems, HRP (DakoCytomation), according to the manufacturer's instructions. The Dako EnVision+ Detection Systems, Peroxidase/DAB (DakoCytomation) was used for chromogenic visualization. The slides were then counterstained with hematoxylin and coverslipped. The expression status of ER, PR, and HER2 was assessed using the same methods as those described previously [21]. ER and PR status was assessed using the Allred scoring method [22]. HER2 expression was evaluated using American Society of Clinical Oncology/College of American Pathologists guideline recommendations [23]. In cases with equivocal HER2 staining (score 2), silver *in situ* hybridiza-

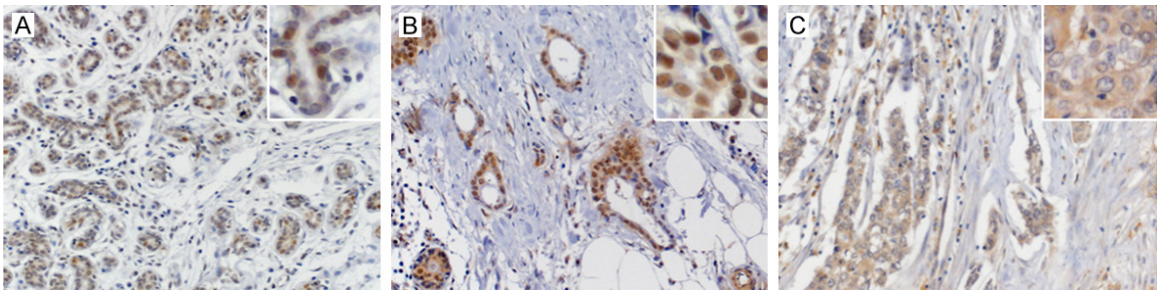

Figure 1. Representative photomicrographs of zinc finger and BTB domain containing 7A (ZBTB7A) immunostaining. A. Nuclear ZBTB7A expression in the ductal epithelium of normal breast tissue. B. High nuclear ZBTB7A expression in breast cancer tissue. C. Low nuclear ZBTB7A expression in breast cancer tissue.

tion was performed to determine HER2 gene status. For ZBTB7A expression, the nuclear and/or cytoplasmic staining was separately assessed. The nuclear reactivity was scored as the percentage of positive nuclei among the tumor cells. The histogram of nuclear percentage score was roughly bimodal with a trough at 65%. Therefore, nuclear expression was stratified into low and high with a 65% cutoff. Cytoplasmic staining was scored as 1 (absent or weak), 2 (moderate), or 3 (strong) and further dichotomized as low (score 1-2) and high (score 3) expression. In IDC cores where ductal carcinoma *in situ* (DCIS) and/or non-neoplastic ducts coexisted, nuclear and cytoplasmic scores for DCIS/non-neoplastic ducts were documented along with those for IDC.

Statistical analysis

The Chi-square test, Fisher's exact test, or linear-by-linear association test was performed to determine the correlation between ZBTB7A expression status and clinicopathological characteristics. Overall survival (OS) was defined as the period from the first tumor resection to disease-specific death. Recurrence-free survival (RFS) was the time between the first tumor resection and the first radiological detection of recurrence or metastasis. Days to last follow-up replaced OS and RFS for censored cases. Univariate and multivariate survival analyses were used to examine the prognostic significance of ZBTB7A expression. Curves for OS and RFS were drawn according to the Kaplan-Meier method, and differences were analyzed using the log-rank test for univariate survival analysis. Multivariate survival analysis was performed for parameters that achieved statistical significance in univariate survival analysis, using the Cox proportional hazards model (95% confidence interval) with a backward stepwise elimination method. Statistical analyses were performed using PASW Statistics for Windows (version 18.0; IBM SPSS, Chicago, IL, USA). Statistical significance was defined as a *p*-value of <0.05.

Results

ZBTB7A expression and its association with clinicopathological characteristics

Representative photomicrographs of ZBTB7A immunostaining in normal breast tissue and IDC are shown in **Figure 1**. ZBTB7A immunoreactivity was observed in the ductal epithelium of normal breast tissue and the peri-tumoral non-neoplastic ducts. In normal breast tissue, most of the ductal epithelial cells showed moderate nuclear ZBTB7A immunoreactivity. The median value of the nuclear percentage score in normal breast tissue was 70%. The myoepithelial cells occasionally displayed nuclear ZBTB7A expression. In IDC tissues, nuclear staining ranged from null to near 100% in different tumors (median, 30%). Applying a cutoff of 65% for nuclear staining, 76 of 196 IDC cases (38.8%) were classified as having high nuclear ZBTB7A expression, and the remaining 120 cases (61.2%) were classified as having low nuclear ZBTB7A expression. Cytoplasmic ZBTB7A staining in IDCs was strong in 28.1% (55/196) and weak-to-moderate in 71.9% (141/196). There was an inverse relationship between the nuclear and cytoplasmic ZBTB7A expression in IDC (P=0.009). In cores where DCIS coexisted with IDC (58 cases), the ZBTB7A expression pattern of DCIS was mostly indistinguishable from that of IDC.

The association between ZBTB7A expression and clinicopathological characteristics of IDC patients was analyzed. Low nuclear ZBTB7A

Table 1. Association between zinc finger and BTB domain containing 7A (ZBTB7A) expression and clinicopathological characteristics of invasive ductal carcinoma of the breast

Characteristic		Total, n	Nuclear ZBTB7A expression, n (%)		*p*-Value
			Low	High	
Age (years)	>40	32	23 (71.9)	9 (28.1)	0.176
	≤40	154	97 (59.1)	67 (40.9)	
Tumor size (cm)	>2	106	73 (58.9)	33 (31.1)	0.017*
	≤2	90	47 (52.2)	43 (47.8)	
Histological grade	1	55	22 (40.0)	33 (60.0)	<0.001*
	2	80	53 (66.3)	27 (33.8)	
	3	61	45 (73.8)	16 (26.2)	
Pathological T stage	pT1	92	52 (56.5)	40 (43.5)	0.227
	pT2	99	65 (65.7)	34 (34.3)	
	pT3	4	2 (50.0)	2 (50.0)	
	pT4	1	1 (100.0)	0 (0)	
Pathological N stage	pN0	106	64 (60.4)	42 (39.6)	0.355
	pN1	57	33 (57.9)	24 (42.1)	
	pN2	17	11 (64.7)	6 (35.3)	
	pN3	16	12 (75.0)	4 (25.0)	
Distant metastasis	Present	18	11 (61.1)	7 (38.9)	0.992
	Absent	178	109 (61.2)	69 (38.9)	
Stage	I	65	35 (53.8)	30 (46.2)	0.126
	II	97	62 (63.9)	35 (36.1)	
	III	33	22 (66.7)	11 (33.3)	
	IV	1	1 (100.0)	0 (0.0)	
ER	Positive	144	74 (51.4)	70 (48.6)	<0.001*
	Negative	52	46 (88.5)	6 (11.5)	
PR	Positive	131	69 (52.7)	62 (47.3)	<0.001*
	Negative	65	51 (78.5)	14 (21.5)	
HER2	Positive	54	38 (70.4)	16 (29.6)	0.105
	Negative	142	82 (57.7)	60 (42.3)	
Triple negativity	Yes	26	25 (96.2)	1 (3.8)	<0.001*
	No	170	95 (55.9)	75 (44.1)	
Recurrence	Yes	29	23 (79.3)	6 (20.7)	0.044*
	No	161	96 (59.6)	65 (40.4)	

ER: Estrogen receptor; PR: Progesterone receptor; HER2: human epidermal growth factor receptor 2; ZBTB7A: zinc finger and BTB domain containing 7A. *Statistically significant.

expression in IDC was significantly associated with larger tumor size (P=0.017), higher histological grade (P<0.001), ER negativity (P< 0.001), PR negativity (P=0.001), triple negativity (P<0.001), and recurrence (P=0.044; **Table 1**). No statistically significant association was found for other clinicopathologic characteristics, including age, pathological T stage, pathological N stage, stage group, or lymphovascular invasion. In contrast with the nuclear ZBTB7A expression, cytoplasmic ZBTB7A expression showed no significant associations with clinicopathological characteristics.

Effects of ZBTB7A expression on outcome of patients with IDC

The median follow-up time was 87 months. The 5-year OS and RFS rate was 96.4% and 88.5%, respectively. Univariate analysis of OS revealed that distant metastasis (P<0.001), advanced stage (P=0.038), and triple negativity (P=0.043) significantly predicted poor OS (**Table 2**). There was no significant difference in OS according to nuclear ZBTB7A expression status (**Figure 2A**). The difference in OS according to the higher histologic grade showed marginal significance (P= 0.063, representatively). Multivariate analysis of OS revealed that distant metastasis was a significant predictor of poor OS (P<0.001). Univariate analysis of RFS revealed that distant metastasis (P<0.001) and triple negativity (P=0.033) were significant predictors of poor RFS (**Table 2**). The difference in RFS according to the nuclear ZBTB7A expression status showed marginal significance (P=0.069; **Figure 2B**). In multivariate analysis of RFS, distant metastasis (P<0.001) and low nuclear ZBTB7A expression (P=0.009) were significant predictors of poor RFS. Low nuclear ZBTB7A expression was found to independently predict RFS (hazard ratio =3.432, 95% confidence interval =1.364-8.637; **Table 2**). Cytoplasmic ZBTB7A expression did not affect OS or RFS.

Discussion

Breast cancer is the most common malignancy in women worldwide. Various histological types

Table 2. Factors predicting shortened overall and recurrence-free survival of patients with invasive ductal carcinoma of the breast

Characteristic	Overall survival			Recurrence-free survival		
	Univariate	Multivariate		Univariate	Multivariate	
	p-Value	*p*-Value	Hazard ratio (95% confidence interval)	*p*-Value	*p*-Value	Hazard ratio (95% confidence interval)
Age >40 years	0.885		Not applicable	0.819		Not applicable
Higher histological grade	0.063	0.069	6.939 (0.857-56.182)	0.551		Not applicable
pT2-4	0.592		Not applicable	0.736		Not applicable
Lymph node metastasis	0.437		Not applicable	0.493		Not applicable
Distant metastasis	<0.001*	<0.001*	65.143 (15.980-265.551)	<0.001*	<0.001*	38.390 (16.464-89.520)
Advanced stage	0.038*	0.703	1.515 (0.179-12.854)	0.106		Not applicable
ER negativity	0.119		Not applicable	0.222		Not applicable
PR negativity	0.143		Not applicable	0.098	0.626	0.780 (0.288-2.115)
HER2 negativity	0.902		Not applicable	0.357		Not applicable
Triple negativity	0.043*	0.179	2.289 (0.683-7.669)	0.033*	0.446	1.409 (0.583-3.404)
Low nuclear ZBTB7A expression	0.843		Not applicable	0.069	0.009*	3.432 (1.364-8.637)

ER: Estrogen receptor; PR: Progesterone receptor; HER2: human epidermal growth factor receptor 2; ZBTB7A: zinc finger and BTB domain containing 7A. *Statistically significant.

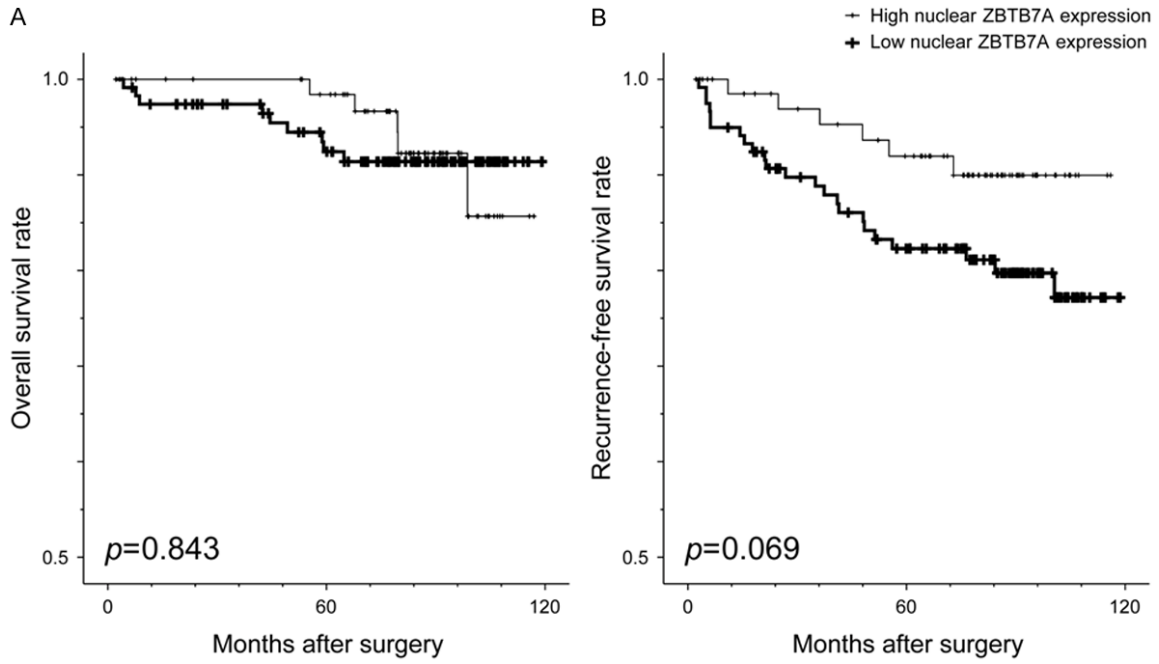

Figure 2. Prognostic significance of zinc finger and BTB domain containing 7A (ZBTB7A) expression in patients with breast cancer. Kaplan-Meier curves illustrating (A) overall survival and (B) recurrence-free survival.

of breast cancer have been reported, with IDC being the most frequently occurring type [24, 25]. The immunohistochemical expression of ER, PR, and HER2 has been widely used for predicting the prognosis of breast cancer and for providing therapeutic strategies [26]. Since Perou et al. [27] reported the molecular features of breast cancer cells in 2000, the improvements in molecular techniques have provided a framework to establish molecular subtypes, namely luminal A; luminal B (HER2-negative); luminal B (HER2-positive), HER2 subtype; basal phenotype; and five negative phenotypes [27-29]. Breast cancer-expressed hormonal receptors, including ER and PR, or amplification of HER2, have been used in various targeted treatment approaches [30, 31]. However, the effort has been devoted to identifying factors of prognostic and therapeutic significance in IDC, one of which is ZBTB7A.

ZBTB7A mRNA and protein have been identified in both normal and cancerous tissues of multiple organs, and the expression of ZBTB7A in cancer has provided ambivalent results. Some researchers have reported that ZBTB7A has unfavorable prognostic effects in several malignancies by suppressing p14ARF or upregulating survivin [7, 10, 11, 13-15]. On the other hand, favorable prognostic effects of ZBTB7A expression have also been reported. Reduced ZBTB7A expression has been associated with worse prognosis through affecting another pathway such as upregulation of MCAM or repression of glycolysis or survivin, implicating its tumor-suppressive role [12, 16-18]. Based on these reports, ZBTB7A appears to play several different roles in malignancy through variable molecular pathways.

In this study, the immunohistochemical expression of ZBTB7A was analyzed in patients with IDC. Low nuclear ZBTB7A expression was significantly associated with aggressive oncogenic behavior including larger tumor size, higher histological grade, and recurrence. In addition, reduced ZBTB7A expression in the tumor cell nuclei correlated with negative ER and PR expression. The triple-negative phenotype was more frequently observed in patients whose tumors showed low nuclear ZBTB7A expression. ZBTB7A expression was not statistically associated with OS. Only distant metastasis was an independent prognostic factor for OS. No clinicopathological parameters influenced the RFS except distant metastasis and triple negativity. Even though RFS of patients with low nuclear ZBTB7A expression was lower than that of patients with high nuclear ZBTB7A expression, the difference was statistically marginally significant. Nevertheless, in multivariate analysis for RFS, low nuclear ZBTB7A expression was found to independently predict RFS, suggesting that ZBTB7A expression is a novel prognostic biomarker for RFS of patients with IDC. Our results agree with those of previous studies demonstrating an association between low ZBTB7A expression and aggressive oncogenic behavior in non-small cell lung cancer, ovary carcinoma, gastric carcinoma, and hepatocellular carcinoma [7, 10, 11, 13].

In conclusion, we demonstrate that low nuclear ZBTB7A expression is associated with unfavorable clinicopathological characteristics including larger tumor size, higher histological grade, negativity for ER and PR, triple negativity, and recurrence in IDC. In addition, there was a significant relationship between low nuclear expression of ZBTB7A and poor RFS. Our data suggest that nuclear ZBTB7A expression is a novel prognostic biomarker for patients with breast cancer.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016-R1D1A1B03935584) and the Medical Research Funds from Kangbuk Samsung Hospital (2016).

Disclosure of conflict of interest

None.

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